PATENT APPLICATION

Antibody Production in Farm Animals

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Title: Antibody production in farm animals.

The invention relates to the field of immunology. More in particular the invention relates to means and methods for antibody production.

Antibodies find many applications in science and medicine. They
5   have a remarkable capability to bind to very specific targets. Moreover, it is fairly straightforward to generate new antibodies against a target. Antibodies can be produced in a variety of ways. For most applications antibodies are produced by so-called hybridoma cell lines that result from the fusion of an antibody producing B-cell with an immortalized cell line. Such hybridoma cells
10 can easily be cultured and the antibody can be harvested from the culture supernatant. Another method for the production of antibodies is the harvesting from serum of immunized animals or the production of antibody fragments by bacteria. Of course many other production methods exist.

15   Although many different methods for the production of antibodies exist, they all suffer more or less from the same problem. The cost of production is relatively high. For hybridoma based cultured systems, large culture systems need to be set up, validated and maintained. When serum is the source, blood needs to be collected and processed. Moreover, systems need
20 to be in place to check for microbial/viral contamination of the produce. The high production costs prevent the implementation of many applications where the cost of the antibody is important. For instance, many diagnostic kits for diseases are not generally available to third world countries.

25   One of the routes travelled to reduce the cost of producing antibodies is using farm-animals. Technology for the breeding of farm-animals is widespread and farm-animal housing is relatively cheap. Several initiatives have been undertaken to produce antibodies in the milk of farm-animals. For example, immunization via the supramammary lymph node was described in

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Guidry et al., J. Dairy Sci. 77(10): 2965-2974 (1994) and in Tomita et al., J
Dairy Sci. 81 (8): 2159-2164 (1998). Immunization via either intranasal or
intramammary routes was described in Woods, J. Am. Vet. Med. Assoc. 173 (5
Pt 2): 643-647 (1978) and in Bohl et al., Infect. Immun. 11(1): 23-32 (1975).

5   Commercial scale implementation has been attempted by immunization via
supramammary lymph node as described in Leitner et al., WO 99/33954 and
via direct injection into the mammary tissue as described in Petersen et al.,
USPN 3,376,198 and in Hasting, USPN 5,017,372. However, thus far it is
possible to obtain only limited amounts of immunogen-specific antibodies in

10   mammary secretion products of farm animals. Best results are obtained in the
colostrum, i.e. the first lacteal fluid that is produced by the female following
birth of a young. Milk produced by the female following the colostrial stage is
called herein mature milk. Colostrum is quite a unique product that arises
from a distinct physiological and functional state of the mammary gland. In

15   ruminants, the principal compositional difference between colostrum and
mature milk is the very high content of colostral immunoglobulin, of which
IgG class makes up 80-90%.

Immunoglobulin (mg/ml)	colostrum	Milk
IgG-total	32-212	0.72
IgG$_1$	20-200	0.6
IgG$_2$	12.0	0.12
IgA	3.5	0.13
IgM	8.7	0.04

McFadden, T.B. et al. (1997) in Milk Composition, Production and
Biotechnology, Welch, R.A.S., Burns, S.R., Popay, A.I. and Prosser, C.G.,
Eds. pp133-152, CAB International, New York).

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Although the antibody levels in colostrum are higher than in mature (normal) milk, the harvest of the colostrum requires exact timing of the birth since the colostrum only lasts for a maximum of two to three days following birth of the young. Moreover, since colostrum is given only for two or three

5   days, the absolute amounts of antibody that can be collected per animal are limited. For the larger, commercial scale production of antibodies a large collection of farm-animals must be held. The requirement for a large collection of farm-animals also necessitates greater numbers of immunizations etc. thus increasing the logistical problems and production costs. Moreover, due to the

10   thickness/viscosity of the colostrum (it is very thick in fat and protein) the down-stream processing of colostrum is problematic.

For decades, various (immunization) attempts have been made to obtain increased secretion of immunogen-specific antibodies via the mammary gland of farm animals. Such attempts were partially aimed at controlling

15   infections in the mammary gland, i.e. mastitis (Guidry et al., J. Dairy Sci. 77(10): 2965-2974 (1994); Pighetti et al., J. Dairy Sci. 78 (3): 528-537 (1995)) as well as at production of large quantities of immunogen-specific antibodies via milk. The antibody levels in mature milk, however, still remain lower (approximately an order of magnitude) when compared to those that can be

20   achieved in colostrum (Hodgkinson et al., WO 98/54226; Hastings, USPN5017372). Accordingly, antigen-specific antibodies employed in most clinical and preclinical studies are colostrum-derived and thus belong predominantly to the IgG class (Tollemar et al., Bone Marrow Transpl. 23: 283-290 (1999); Bostwick et al., USPN5,773,000; Cordle et al., USPN

25   5,260,057).

The present invention provides a harvested mammary secretion product comprising an antibody specific for an antigen, said mammary secretion product obtainable by a method comprising

30   - hyperimmunizing a farm-animal for said antigen and

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- administering said antigen to a mammary gland and/or a supramammary lymph node of said animal and

- harvesting said mammary secretion product from said farm-animal. Said mammary secretion product surprisingly contains much higher levels of

5   antibody specific for said antigen (herein called antigen-specific antibody) than secretion products obtained via conventional methods in the art. Moreover, it was observed that using the methods of the invention it was possible to obtain substantial amounts of antigen-specific antibody in the mature milk of the lactating animal. Indeed, the levels of such antigen-specific antibodies were in

10   the range of those that can be raised in colostrum. By hyperimmunizing is meant that the animal produces a supranormal level of antigen-specific antibodies in the blood or in mucosal secretion, not in lacteal secretion. A supranormal level means that the amount of antibodies in the animal body is higher than it would be without administration of the antigen, or after a single

15   administration of the antigen. Preferably, said hyperimmunizing comprises administering said antigen to an airway of said animal. With said airway administration it was possible to obtain prolonged production of substantial amounts of antibody in the milk of the animal without necessitating additional booster administrations of said antigen to said animal. However, booster

20   administrations of said antigen to said animal may be performed in cases where the levels of antigen-specific antibody are found to drop after some time. Preferably, said airway administration comprises intra-nasal administration of said antigen. Intra-nasal administration is relatively easy to perform and airway administration has been observed to induce higher levels of antigen-

25   specific antibody in the mammary secretion product and more sustained production of the antibodies in the milk of the animal over time. Preferably, said mammary secretion product is milk. Milk is easy to collect and since the antigen-specific antibody production in the milk is sustained over time it is possible to select a group of animals that have the best levels of antigen-

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specific antibody in the milk thus improving the overall performance and logistics of the procedure.

5   The invention thus demonstrates the feasibility of producing a unique lacteal fluid in a prolonged manner. The characteristics of such a fluid are: a) said fluid has the characteristics of colostrum as far as antigen-specific antibody levels are concerned; b) yet the overall compositional characteristics of said fluid are comparable with those of milk. By the characteristics of colostrum as far as antigen-specific antibody levels are concerned is meant

10   herein, that the amount and/or kind of antigen-specific antibody is more comparable to colostrum than to milk. For instance, the amount of antigen-specific antibody in the fluid has to be higher than the amount of antigen-specific antibody normally obtained in milk. In that case the amount of antigen-specific antibody in the fluid is at least in part comparable to the

15   amount of antigen-specific antibody in the colostrum, because both amounts are higher than the amount of antigen-specific antibody normally obtained in milk.

        It is clear that any immunization schedule resulting in an animal that is hyperimmunized for said antigen can be combined with administration

20   of antigen to a mammary gland and/or supramammary lymphnode, to obtain the result. There is no real need to assess whether an animal is hyperimmunized before administration of antigen to a mammary gland and/or supramammary lymphnode is done. One can easily determine the antibody level in mammary secretion product of said animal. A low level of antibody in

25   said secretion product indicates that said animal was not hyperimmunized and additional administration of antigen is required to achieve hyperimmunization. A group of animals treated with a method of the invention may contain individuals that do not respond or not respond to a sufficient extend. Such non-responders can be excluded from the collection of

30   milk. With a method of the invention it is possible to obtain at least 25 %

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responders in a group. Frequently at least 50% of the animals treated with a method of the invention respond to exhibit prolonged high antigen-specific antibody production in the milk of a lactating farm-animal and/or mammal.

5   Antigen to which said antigen-specific antibody is raised can be any compound or collection of compounds capable of eliciting an immune response. Typically said antigen comprises a protein or a functional part, derivative and/or analogue thereof. Antigen administered to said animal does typically not differ from administration to administration to the animal. However, this does not necessarily have to be true. It is possible to use different

10   manifestations of antigen, for instance different proteins, as long as different manifestations comprise at least one part that is immunologically the same. By immunologically the same is meant that an antibody is capable of recognizing different manifestations of said antigen. Antigen may also be administered through administering nucleic acid encoding said antigen or

15   functional equivalent thereof to said animal. Administered nucleic acid can be expressed by cells of said animal to which said nucleic acid has been delivered. Expression of said antigen or functional equivalent in cells of the animal leads to the mounting of an immune response. This technology is also in the art referred to as nucleic acid vaccines.

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Without being bound by theory it is thought that a high general immune response is required to provide a substantial pool, circulation or reservoir of antigen-specific antibody producing cells. A mammary gland and/or supramammary lymphnode immunization is required to attract cells

25   from the pool, circulation or reservoir to the mammary tissue such that secretion of the antigen-specific antibody to the mammary excretion product is enabled. It is preferred that the generation of the pool, circulation or reservoir is achieved with an immunization schedule resulting in a high mucosal immune response since in this way at least part of the pool, circulation or

30   reservoir may be primed toward the mammary gland. Thus facilitating earlier

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and more pronounced secretion of the antigen-specific antibody upon mammary gland and/or supramammary lymphnode immunization. Preferably, however, the pool, circulation or reservoir is generated through an immunization schedule resulting in a high mucosal and/or systemic immune
5   response.

An immunization schedule comprises one or more administrations of antigen to a farm-animal. An immunization schedule resulting in a high mucosal and/or systemic immune response is preferably at least in part achieved through inhalation of antigen by said animal. The composition for
10   inhalation is of course preferably administered such that antigen in the composition is distributed throughout the major part of the airway of said animal. Airway administration is preferably achieved in the form of aerosols. Preferably, said airway administration is performed through intra-nasal administration. Preferably the immunization schedule resulting in a high
15   mucosal and/or systemic immune response comprises at least two airway administrations of a composition comprising the antigen. More preferably, it comprises at least four airway administrations of a composition comprising the antigen.

With means and methods of the invention both a mammary gland
20   immunization and a supramammary lymphnode administration of a composition containing the antigen have the effect of enabling the secretion of large amounts of antigen-specific antibody in the milk of a farm-animal. Preferably at least one administration is performed in the supramammary lymphnode. Preferably, at least two administrations are performed in the
25   supramammary lymphnode. Supra-mammary lymphnode administration results in a higher and earlier surge of antigen-specific antibodies in the milk.

Means and methods of the invention are suited to obtain high and prolonged antigen-specific antibody production in the milk of any lactating mammal. Preferably, said animal is a farm-animal. Farm animals are animals
30   that are used on a commercial basis by man, be it for the production of milk,

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meat or even antibodies. Farm-animals already used for the commercial scale production of milk are preferred for the present invention since for these animals special lines and/or breeds exist that are optimized for milk production. Preferably, said farm-animal is a cow or a goat. More preferably

5   said farm-animal is a cow.

Using the methods of the invention it is possible to obtain high levels of antigen-specific antibody in any mammary secretion product such as for instance the colostrum or milk.

Antigen-specific antibodies in the milk can be of any immune class.

10   However, preferably said antibodies are of the IgG and/or IgA class. More preferably, said antibodies are of the IgA class.

Milk, containing antigen-specific antibodies is preferably collected by milking the animal. Milk thus collected can either be used directly, or the milk can be further processed, for instance to purify antigen-specific

15   antibodies. Methods for the (partial) purification of (antigen-specific) antibodies from milk are present in the art and need not be listed here.

Antigen-specific antibodies of the invention can be used for almost any purpose. Preferably said antibodies are used for a purpose that polyclonal antibodies are used for in the art. However, using purification methods in the

20   art it is also possible to obtain essentially monoclonal antibodies, for instance through immunization with an antigen comprising essentially only one immunogenic part. Essentially monoclonal antibodies can also be produced in other ways for instance through immune purification of antibody using a peptide, comprising essentially only one binding region for antibody. Antigen-

25   specific antibodies (purified from the milk or not) are preferably used for the preparation of a medicament. Such antibodies can be used for instance for the preparation of medicaments for the treatment of skin wounds, for instance with an antibody specific for a skin bacterium. Such antibodies are also particularly suited for the treatment of disease that are caused by pathogens

30   in the gastro-intestinal tract. In general, the antigen-specific antibodies from

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the milk of a farm animal can be used in any (at least in part) immune protected area of the human body. Such areas are for instance the gastro-intestinal tract, the respiratory tract, the urogenital tract, the eye, the mouth and the skin. However, such antibodies can also be used to bind antigens that
5   can be reached by the blood flow in a patient, for instance following systemic administration of the antibodies to said individual. Considering that the individual to be treated is usually a human, and the antibodies are derived from a farm-animal, it is expected that the immune system of the individual will respond to the administered antibodies, especially when administered
10   systemically. However, many applications of such systemic administration of antibodies are still possible. For instance, when the individual has as yet not mounted an immune response to the antibodies (as for instance is the case in a first use in said individual) or when the immune response has faded (over time).
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        Antigen-specific antibodies (purified from the milk or not) can also be used for the preparation of for instance a food product. Many bacterial but also viral microbes can be present in the gastro-enteric tract. Some of those microbes can be present sub-clinically. With a method of the invention
20   antibodies can be generated that are specific to one or more of said microbes. Such antibodies can be used to prepare a food product or other product. By eating the product, antibodies toward said one or more microbes are released in the gastro-enteric tract thus at least in part preventing said microbes from multiplication.
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        Considering that the antibodies are produced in a farm-animal, the antibodies will typically be specific for antigens that are not naturally present in said animal (non-self). Thus generally antibodies can be produced against any antigen that is foreign to the animal. Typically such antibodies comprise
30   specificity toward bacteria, viruses and toxins thereof. However, antibodies

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can also be raised against mammalian antigens when there is sufficient divergence between the antigen administered and the homologue of the antigen in the farm-animal. Although less straightforward it is also possible to generate an antibody response to at least some self-antigens. Several methods
5   to achieve that result exist in the art.

One or more of the immunization schedules may further include administration of one or more other compounds capable of stimulation and/or modulating an immune response of the farm-animal. Suitable adjuvants are known in the art and may include among others Freund's incomplete adjuvant,
10   aluminum hydroxide, saponin/cholestrol based immune-stimulating complexes ISCOMS, and the glyceride-polysorbate based adjuvant 'RhinoVax' (Jakobsen et al. Infect. Immun. 67: 4128-4133 (1999). Preferably, said adjuvant is capable of modulating an immune response toward a mucosal immune response. Non-limiting examples of such compounds are $1\alpha,25(OH)_2D_3$, cholera
15   holo toxin, toxin A and toxin B of *Clostridium difficile*, cytokines such as IL-5, IL-6, IL-12 or TGF-3, unmethylated C_pG (bacterial DNA) sequences with phosphodiester backbone. Commercially available alternatives to Freund's complete adjuvants include Ribi adjuvant system (RAS), Titer Max, Syntex Adjuvant Formulation (SAF), Elvax 40W, Montanide, AdjuPrime, Gerbu
20   Adjuvant and Super Carrier. Antigens can also be coprecipitated with L-tyrosine or absorved to nitrocellulose for slow release. Of course also combinations of adjuvants can be used.

Methods of the invention may be used to elicit an immune response against the antigen in the farm-animal. However, it is of course also possible
25   to use the methods of the invention to amplify an already existing immunity toward the antigen in the farm-animal.

The farm-animal can be immunized against one antigen, however considering that a polyclonal immune response is generated, antigen-specific antibodies in the milk typically comprise specificity toward two or more
30   different parts of the antigen. When antigen is used that comprises a complex

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mixture of different compounds, many antibodies comprising specificity for different parts of the antigen will be generated and be present in the milk of the animal. It is of course easy to repeat a method of the invention for another antigen one or more times and thus generate mammary secretion product

5   comprising set of antibodies for one or more antigens or several cross-linked antigens.

Immune responsiveness of farm animals to a given antigen can be further enhanced via genetic manipulation to the animals. For example, each

10   of the numerous biochemical steps that are critically involved in the process from B-cell activation to antibody secretion in the mucosal surfaces can be optimized via genetic modification. Non-limiting examples are: increased expression of specific MHCantigen molecules for efficient antigen presentation, increased expression of J-chain for increased IgA dimer formation, and/or

15   increased expression of homing receptors for increased migration of activated lymphocytes to the mammary tissue. Expression of numerous cytokines and lymphokines which are involved in the process can also be manipulated to optimum levels for maximum antibody secretion. Furthermore, animals that are proven to be high responders to a given antigen can be cloned, so that upon

20   immunization of the cloned animals, high antigen-specific antibody yields are ensured.

In one embodiment the antigen comprises a collection of compounds harvested from a culture of *Clostridium difficile*. This collection of compounds

25   contains several proteins among which formaldehyde-inactivated *C. difficile* (VPI10463) cells, formaldehyde-inactivated spores of said bacterium and formaldehyde-inactivated *C. difficile* toxoids which are toxin A and toxin B. Antibodies raised against this collection will typically comprise specificity toward more than one protein of *C. difficile*. Preferably, said antibodies

30   comprise antibodies specific for spores of *C. difficile*. Preparations of such

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antibodies can be used for the preparation of a medicament for the treatment of diseases associated with *C. difficile* infections. Administration to an individual is preferably performed such that antibodies are delivered to the gut. Several methods exist in the art to allow for such delivery, for instance,

5   antibodies can be contained in pills that release their content to the gut. In a preferred embodiment of the invention the antibodies of the invention comprise specificity toward spores of the *C. difficile*. Such antibodies are particularly useful to at least in part prevent recurrence of *C. difficile* related disease. Typically, *C. difficile* related disease is treated with medication

10   directed toward removal of bacteria of *C. difficile*. Such medication can consist for instance of the administration of antibiotics and/or antibodies directed toward one or more bacterial components of *C. difficile*. Although this treatment is usually successful in curing the patient from symptoms, it is often (20% of the cases) not sufficient to prevent recurrence of the disease in the

15   patient. Recurrence can at least in part be prevented by delivering to the gut of the patient antibodies with a specificity toward spores of *C. difficile*. Binding of said antibodies to at least part of the *C. difficile* spores in the gut at least in part reduces the propensity with which said spores can hatch. Thus preventing at least in part, recurrence of *C. difficile* related disease in the patient. Thus

20   the invention also provides a composition comprising an antibody capable of binding to a spore of *C. difficile*. Further provided is the use of a *C. difficile* spore specific antibody for the preparation of a medicament. Preferably, said antibody is used for the preparation of a medicament for the treatment of *C. difficile* related disease.

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Lactating farm-animals can suffer from bacterial and/or viral infections in the mammary gland. Such infections can at least in part be treated by enabling antigen-specific antibody secretion into the secretion compartment of the mammary gland. One of such diseases is mastitis. The

30   invention therefore further provides a method for the treatment of microbial

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infection of a mammary gland in an animal comprising administering to said animal an antigen such that a high immune response is obtained and administering said antigen to a mammary gland and/or a supramammary lymph node of said animal, wherein said antigen comprises at least a part of

5   said microbe or a functional derivative and/or analogue thereof. Said immune response may be a systemic immune response. Preferably, said immune response is a general (both systemic and mucosal) immune response. Preferably, said animal is suffering from or at risk of suffering from mastitis.

The invention further provides a use of an antigen for the preparation

10   of a medicament for the treatment of a microbial infection of a mammary gland, wherein said medicament comprises a composition for airway administration of said antigen and a composition for mammary gland and/or supramammarylymphnode administration of said antigen. Preferably, said microbial infection causes mastitis. Said medicament may also be suitable for

15   the treatment of a human.

Mastitis is a disease that causes the transient and sometimes permanent loss of milk production. Loss of milk production can be induced by the disease itself or by the use of antibiotics that affect the milk production, or both. Animals suffering from mastitis get very sick and sometimes die.

20   Mastitis considerably effects the amount of labor and costs of production for a farmer.

In another aspect the invention provides milk obtained from a lactating farm-animal comprising at least 0.5 µgram/ml antigen-specific

25   antibody. Preferably, said milk comprises at least 15 µgram/ml antigen-specific antibody. More preferably, said milk comprises at least 50 µgram/ml of antibody against which said animal has been immunized with a method of the invention.

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Administration of antigen to said animal can be achieved through means known in the art. Typically antigen is administered in a range that for a protein of approximately 10 kD varies between 5-500 μgram. Antigen based on whole (inactivated) cells and/or spores, is typically administered such that

5   the equivalent to the range of 10^8 and 10^{11} cells and/or spores are provided to said animal. Other concentrations and/or amounts can be used as well, depending on the size of the animal, the immunogenicity of the antigen and/or adjuvants and other variables known in the art.

10   Much evidence exists in the literature that pathogen-specific antibodies obtained from colostrum and milk of cows are effective for prevention and treatment of gastro-intestinal infections caused by various pathogens. In spite of promising indications, however, product development has not yet been widely materialized due mainly to unavailability of sufficient

15   amounts of pathogen-specific antibodies.

It is relatively easy to obtain high levels of antigen-specific antibodies from colostrum of cows after immunization via conventional methods. However, secretion of colostrum lasts only for 2 to 3 days after each calving and as soon as they enter into a mature lactation period, levels of such

20   antibody drop close to nil. In the present invention, we describe immunization methods that result in secretion of high levels of antigen-specific antibodies not only in colostrum but also in mature milk of lactating farm-animals for a sustained period of time. As we show in the examples, using a method of the invention it is possible to obtain mature milk from an animal, comprising

25   antigen-specific antibody in a quantity which is at least half as much as which is in average possible to obtain in the colostrum of said animal using conventional methods of the art.

Therefore, in one aspect the present invention provides mature milk obtainable from a mammal, comprising an antigen-specific antibody in a

30   quantity of at least 50 percent of the average quantity of said antigen-specific

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antibody obtainable in colostrum from said mammal, wherein said colostrum is obtained after immunization of said mammal against said antigen. Preferably, said antigen-specific antibody consists of IgA, because IgA forms dimers which are very well capable to form complexes.

5   By an average quantity of said antigen-specific antibody obtainable in colostrum is meant herein a quantity which is the average of yields of antigen-specific antibody, obtained from colostra from different unselected animals, which have been immunized against said antigen.

          A yield of at least 50 percent is an important improvement, because
10   in the prior art only yields of antigen-specific antibody in mature milk of about one tenth compared to yields obtained from colostrum was possible. However, as we show in the examples, the present invention provides higher yields of IgA in mature milk than the yields that were obtained in colostrum using conventional methods of the art. Thus, in a preferred aspect the invention
15   provides mature milk according to the invention, wherein said percentage is at least 100 percent, more preferably at least 200 percent.

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EXAMPLES

MATERIALS AND METHODS

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DUKORAL⊚, an oral cholera vaccine approved for human use, was purchased from SBL Vaccin AB, Stockholm. Each ampule consists of formalin-inactivated *Vibrio cholera* and cholera toxin subunit-B (CTB), at concentrations of 10^{11} cells and 1 mg protein, respectively, in 3 ml phosphate-

10   buffered saline, pH 7.4 (PBS).

Clostridium difficile **whole-cell.** *C. difficile* whole-cell was prepared and inactivated as described previously. Briefly, *C. difficile* VPI 10463 was grown in BHI medium at 37°C under an anaerobic atmosphere for 36 h. Cultures were centrifuged and cells were washed three times with PBS. The

15   resulting pellets were resuspended in PBS containing 1% (vol/vol) formaldehyde and kept at 4°C until use. Before each immunization, excess formaldehyde was removed by two washes with PBS. Inoculation of the equivalent to 10^9 *C. difficile* CFU (a cell suspension with an optical density of 1.0 at 550 nm at 1 cm light path) into BHI medium yielded no growth after

20   36h at 37°C in an anaerobic atmosphere.

C. difficile **toxoid.** *C. difficile* culture filtrate was prepared and inactivated as described previously. Briefly, *C.difficile* VPI 10463 was grown in proteose peptone-yeast extract medium for 48 h at 37 °C and inactivated by adding formaldehyde to result in a concentration of 1% (vol/vol) and incubating

25   it at 37°C for 1 hour. The supernatant was filter sterilized, washed three times with PBS by ultrafiltration (Amicon, 30 KDa), concentrated 10 times in a 500 ml cell concentrator and stored at −20°C until use.

Animals. Gestating Holstein-Frisian and MRY dairy cows were maintained according to generally accepted dairy management practices in the

30   Netherlands. In experiments described in this communication, mostly

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Holstein-Frisian cows were employed. Additionally, for a first goat experiment, pregnant goats were selected and maintained in a separate farm, also according to generally accepted management practices. For a second goat experiment pregnant Saanen goats were selected and maintained in a

5 separate farm, also according to generally accepted management practices.

Immunization routes and regimens. Cows: For intra-muscular (IM), intra-udder (IUDR) or intra-supramammary lymph node (LMF) immunization, a 2 ml of vaccine preparation in PBS was administered via direct injection into the respective tissues. For intra-nasal (IN) immunization,

10 a 2 ml vaccine preparation was sprayed, while forced to face upwards, into one of the nostrils of cows through a nozzle that was attached to a syringe. For immunizations with the cholera vaccine, DUKORAL was diluted in PBS so that each 2 ml contains 6.5×10^9 V. cholera plus 66 µg of CTB. In cases of the C. difficile (CD) vaccine, the toxoid and the whole-cell preparations were mixed

15 and diluted in PBS to result in 5×10^{10} inactivated C. difficile plus 5.5 mg protein of culture supernatant in 2 ml.

First goat experiment: Vaccines that were prepared for cows were further diluted (to 50%) with an equal volume of PBS. For each immunization, 2 ml was used, with the exception of LMF where only one ml was used.

20 Second goat experiment: Vaccines that were prepared for cows were further diluted (to 50%) with an equal volume of PBS. For each immunization, 2 ml was used, with the exception of LMF where only one ml was used. For intra-nasal (IN) immunization 1 ml vaccine preparation was sprayed into each nostril. The vaccine preparation used for the goats in the second goat

25 experiment was a combination of Dukoral and CD vaccine.

Sample collection and serum and whey preparation. Normally, 5 ml of milk sample from each quarter was collected weekly. When desired, five ml of blood sample was also collected from the tail vein. Whey was prepared

30 from milk samples as described previously. Briefly, fat is removed via

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centrifugation at 4 °C for 15 min at 4,300x g in a MSE Mistral Centrifuge 6000. Casein is removed by acid precipitation at pH 4.6 by adding sodium acetate and acetic acid followed by centrifugation at room temperature for 15 min at 3,500 x g. Blood was left to clot overnight at 4°C and the serum was
5   obtained by centrifugation.

      Assay. Indirect ELISA was carried out to measure immunogen-specific IgG and IgA levels in whey and serum samples. In the beginning (for samples from cows immunized with DUKORAL), ELISA was carried out manually but later (for samples from cows immunized with the CD vaccine and samples
10 from goats immunized with DUKORAL and the CD vaccine), the ELISA robot BioTek OMNI was employed. In both cases, microtiter plates (Greiner 655902, Greiner) were coated with either CTB or inactivated *C. difficile* whole-cell at concentrations of 0.3 μg CTB or 2 x 10^7 cells, respectively, per well. Coating was accomplished by incubation for 2 h at 37°C with CTB or for 2 h at 70°C
15 with *C. difficile* whole-cell. The plates were washed after each incubation step with PBS containing 0.05% Tween 20. The wells were blocked for 1 h at room temperature with 2% gelatin in PBS. Samples were diluted in PBS in duplicates and were incubated for 1 h at 37°C. For the measurement of antigen-specific bovine antibodies in collected samples, digoxigenin-labelled
20 monoclonal anti-bovine IgA or anti-bovine IgG antibodies were prepared in the lab and were used as the secondary antibodies. For the first goat experiment, for the measurement of antigen-specific goat antibodies in collected samples, monoclonal anti-goat antibodies, instead of anti-bovine antibodies, were employed as the secondary antibodies. For detection, horseradish peroxidase-
25 labelled goat anti-Digoxigenin-POD, Fab fragment (Boehringer Mannheim) was used. ABTS, 2,2'-Azino diethylbenzothiazoline sulfonic acid (Sigma) was used as the substrate for horseradish peroxidase. The optical density, after incubation for 30 min at 37°C, was measured at 415 nm with Bio-Tek El$_x$800 reader. For the second goat experiment, for the measurement of antigen-
30 specific goat antibodies in collected samples, digoxigenin-labelled monoclonal

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anti-goat IgG antibodies (Sigma) and polyclonal swine anti-goat IgA(Fc) antibodies (Nordic Immunology) were employed as the secondary antibodies. For detection, horseradish peroxidase-labelled goat anti-Digoxigenin-POD, Fab fragment (Boehringer Mannheim) was used, except for the detection of goat

5   IgA antibodies. In this case horseradish peroxidase-labelled rabbit anti-swine IgG (Nordic Immunology) was used as detection antibody. ABTS, 2,2'-Azino diethylbenzothiazoline sulfonic acid (Sigma) was used as the substrate for horseradish peroxidase. The optical density, after incubation for 30 min at 37°C, was measured at 415 nm with Bio-Tek Elx800 reader or Bio-Tek OMNI.

10   Titers. Levels of immunogen-specifc Ig in samples were expressed in units against the standard of 1000 units/ml. The standard is whey preparation from colostrum of cows that had been immunized with either Dukoral or with the CD vaccine. For the second goat experiment the standard for the goat assays was a whey preparation from colostrum of goats immunized with

15   Dukoral and CD vaccine.

Measurements of total Ig in milk. Total Ig content in each whey sample prepared from immunized and unimmunized milk was measured using high-pressure gel-permeation liquid chromatography.

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RESULTS

Generation of standards for antigen-specific antibody assays.
Cows in their late pregnancy were immunized four times, with a three-week
5   interval inbetween immunization, before calving. For each immunization,
combinations of intra-nasal (IN) and subcutaneous (SC) routes were employed.
The immunogen consisted of an equal volume of both vaccines (DUKORAL and
the CD vaccine) supplemented with cholera toxin ($50\mu g$/cow/immunization) for
the IN route and Freund's incomplete adjuvant (equal volume of the
10   immunogen) for the SC route. From each cow, colostrum of the first milking
was collected and whey was prepared. After necessary dilutions (anti-CTB IgA
320 x; anti-CTB IgG 8,000 x; anti-CD IgA 130 x; and anti-CD IgG 8,000 x)
with PBS, ELISA was performed. The optical density measurements at 415
nm are shown in Figure 1 (anti-CTB antibodies) and in Figure 2 (anti-CD
15   antibodies). The whey preparations were pooled and used as standards.

**Anti-CTB Ig levels in milk of cows after immunization with the
cholera vaccine, Dukoral.** With the goal of obtaining high levels of
immunogen-specific Ig in the milk of mammals for a sustained period of time,
20   various doses of Dukoral as an antigen were tested in lactating cows using
various immunization routes. For example, combinations of subcutaneous
(SC), intra-udder (IUDR), intra-supramammary lymph node (LMF), intra-
vaginal (IVG) and intra-peritoneal (IP) routes were explored with
concentrations of DUKORAL ranging from 50 fold dilution to undiluted ones.
25   Most of the cases, the anti-CTB IgA and IgG titers in the milk were extremely
low (Figure 3 and Figure 4), when compared to our standard of 1000 units/ml.
In a few cases, where the levels reached to a few hundred units, they could not
be sustained for much longer than one week. Moreover, the time of appearance
of such sporadic peaks was not predictable with respect to the time of
30   immunization.

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Anti-CTB Ig levels in milk and blood of cows after nasal spray followed by intra-mammary immunization. A group of six cows in their mature lactation were intra-nasally (IN) immunized once a week for five

5   consecutive weeks with the cholera vaccine, Dukoral. During this five-week period of time, levels of CTB-specific IgA and IgG in whey of the milk and blood were monitored. The low levels (ranging from nil to about 50) in the whey were not significantly different from the results obtained from previous experiments (Figure 5 and Figure 6). Anti-CTB IgG levels in the blood,

10   however, continued to increase with repeated IN treatments (starting from about 10 to about 1000 units at the end of the 5 immunization session), whereas CTB-specific IgA in the blood was under the detectable level throughout the experimental period. A single boost treatment, however, brought interesting changes to these results in number of aspects (Figure 5

15   and Figure 6). The boost treatment, which was given 6 weeks after the last IN immunization, consisted of one IM, one IUDR in the rear right quarter, and one LMF also in the rear right quarter, 2 ml each. The measurement of anti-CTB IgA and anti-CTB IgG titers over a five months period is shown in Figure 5 and Figure 6. A few conspicuous changes which resulted from the intra-

20   mammary boost immunization are the following: i) Both anti-CTB IgA and anti-CTB IgG responses were observed in all (6/6) animals. ii) In comparison to the titers in the standard (1000 units/ml), IgA responses were very strong, reaching to about 300-500% of the standard levels in some cows. IgG responses were lesser than those of IgA were, but still very high (reaching to 30% of the

25   standard in high responders). iii) IgA responses were significantly longer lasting than those of IgG were. iv) Although there was large variation among animals in immune responses, the immunization method employed here could bring the responses in a synchronous manner among animals. v) IgA responses were more or less limited to the immunized quarters whereas IgG responses

30   generally were spread to all four quarters.

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Anti-*C.difficile* (whole-cell) Ig levels in the milk after nasal spray followed by intra-mammary immunization. The interesting observations made in the previous experiment stimulated us to extend the experiment with

5   a different immunogen. Thus, an experiment was carried out with the CD vaccine with a few modifications to the protocol used above. The changes employed in the new protocol were intended for evaluation of the importance of the number of IN immunizations before an intra-mammary injection as well as importance of the interval between IN and intra-mammary immunizations.

10   Each cow in a group of 10 in their mid-lactation received one 2 ml IN and one 2 ml IM immunization. Measurements of anti-CD (whole-cell) IgA and IgG levels in samples collected after the priming, but before the boost, showed expected results: barely detectable (Figure 7). Three weeks after the priming, each cow received boost treatments, which consisted of one 2 ml IN, one 2 ml

15   IUDR in the rear right quarter, and one 2 ml LMF also in the rear right quarter. Milk samples were continued to be collected weekly and measurements for levels of anti-CD (whole-cell) were made using ELISA. A synchronous surge of immunogen-specific IgG, ranging from 130 to 430 units/ml (against our anti-CD standard of 1000 units/ml) depending on the

20   cow, was observed in the first week after the boost treatments (Figure 7). As was observed in the case with the cholera vaccine, anti-CD IgG secretion was evenly distributed among the four quarters and the high level secretion was relatively short lived, declining to about 50% levels in less than a two-weeks period. Unlike the immediate IgG responses after the boost, anti-CD IgA

25   responses were slow to appear and could be detected more than a month later (Figure 8), at which time seven cows (out of 10) were selected out and reimmunized. The reimmunization began with two IN immunizations one week apart. Two weeks thereafter, each cow received boost immunizations of one IN and two LMF immunizations (one for each rear quarter). Unlike in the

30   case of IgG response, and to our surprise, anti-CD IgA response was not

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synchronized among immunized cows. (Synchrony of anti-CD IgA response could later be restored when combination of LMF and IMA routes was applied as a boost (see the experiment for Figure 11). Another unexpected result in this experiment is that the strong quarter-specificity, which was observed in

5   the case of anti-CTB IgA response, was no longer observed, i.e. the immunized quarter (rear right) was not necessarily the one which secreated the highest level of anti-CD IgA.

Relative importance of immunization routes. In order to evaluate

10   the relative contribution of each immunization route to the final outcome of antibody secretion in milk, 8 cows were immunized with the CD vaccine. As expected, no anti-CD antibodies were detected in the milk after two-consecutive immunizations via the IN route, two weeks apart from one another (Figure 9 and Figure 10). One week after the second IN, all of the cows

15   received the third IN in addition to IMA immunization in the front quarters only (on 5/22/2000). One week thereafter (5/28/2000), anti-CD IgGs were detectable in all four quarters, but were extremely low (Figure 9). Anti-CD IgA, however, was not detectable in all four quarters (Figure 10). The 8 cows then were divided into two groups. Four of them (group 1) received the CD

20   vaccine and the other four (group 2) received saline via the LMF route (on 5/29/2000). One week thereafter, there was a noticeable increase in anti-IgG secretion, only in group1 implicating that IMA route maybe relatively unimportant to secretion of the IgG class (Figure 9). As expected, there was no quarter specificity for the secretion. As for the IgA class, however, significant

25   levels of anti-CD antibody secretion were observed in both groups, even in the saline treated group (Figure 10), although the latter decreased rapidly with a passage of time. The data implies that the LMF route plays the major role for IgA secretion but so does the IMA route to a lesser extent. Again (in addition to the previous experiment), there was an apparent disappearance of quarter

30   specificity. This observation appears independent of routes of immunization

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and thus must be the result of the vaccine used, i.e., attenuated toxin A of CD, attenuated toxin B of CD and/or inactivated CD cells. The observation is potentially important because it can lead us to a solution to an undesirable logistical problem of having to collect milk from only rear quarters of cows.

5 (Immunization of the front quarters of a cow via the LMF route is not possible without a surgery). The overall levels of anti-CD antibody, both IgA and IgG, in this experiment (Figure 9 and Figure 10) were at least an order of magnitude lower than those previously observed. The results clearly indicates that it is highly desirable that boosting via the LMF route acompanies other

10 mucosal route(s) such as IMA, intra-vaginal, intra-rectal and/or preferably intra-nasal. One such example where cows were immunized via the LMF and IMA routes after priming via the IN route is shown in Figure 11 (anti-CD IgA) and in Figure 12 (anti-CD IgG).

15 **Levels of total Ig concentration in the milk of immunized cows.** Using the methods described in this communication, antigen-specific antibody levels could be reached (close) to the range normally found in the colostrum of immunized cows. Since the total Ig concentration in colostrum of unimmunized cow is approximately two orders of magnitude higher than that in mature

20 milk, an obvious question is what would be the total Ig concentration in the milk of immunized cows. Measurements carried out using high-pressure liquid chromatography showed that total Ig amounts in immunized milk were not significantly different from those in unimmunized milk, which in average was 0.3 mg/ml. The relative amount of total Ig in immunized and unimmunized

25 milk is shown in Figure 13 (For immune responsiveness of each cow see Figure 8). The value of the control group is that of a pooled one (of 9 samples).

Immune- responsiveness of other mammals than cows. In order to test applicability of the same immunization method to other mammals for

30 similar results, goats were chosen: they are abundantly available and each can produce up to 2,000 litres of milk. Automatic milking systems also are

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available and methods of cheese making from their milk, thus whey making, as well as information on their disease status and handling is widely known.

First goat experiment:

5   Ten goats were immunized three times via IN routes, three weeks apart between immunizations. Two weeks after the third IN, each goat received three immunizations one each via IN, IMA and LMF route. Measurements of levels of anti-CTB IgG, anti-CTB IgA, anti-CD IgG and anti-CD IgA in whey samples showed the similar general pattern of the immune response observed

10   in the milk of immunized cows.

Second goat experiment:

Generation of standards for antigen-specific antibody assays.

Goats in their late pregnancy were immunized four times, with a three-week

15   interval between immunizations, before birth of the kids. Immunization routes used combined intra-nasal (IN) and subcutaneous (SC) ways of administration. As immunogen an equal volume of Dukoral and CD vaccine was used supplemented with Diluvac Forte (1:1) (Intervet) for the SC route. Colostrum of the first milking was collected and whey was prepared. ELISA was

20   performed on the samples after dilution with PBS (anti-CTB IgA 100x; anti-CTB IgG 250,000x; anti-CD IgA 50x; anti-CD IgG 16,000x). Figure 14 (anti-CTB antibodies) and Figure 15 (anti-CD antibodies) show the optical density measurements at 415 nm. The whey preparations were pooled and used as standards.

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Anti-CTB Ig levels and anti-CD Ig levels in milk after nasal spray followed by intra-mammary immunization.

A group of four goats in their lactation period were intra-nasally (IN) immunized with 2ml vaccine (a combination of Dukoral and CD vaccine). Two

30   weeks after the first IN, all goats received a second IN immunization. Two weeks after the second IN immunization, each goat received three combined

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immunizations via IM, IUDR and LMF route. Milk samples were collected during a 12-weeks-period and the immune response (anti-CTB Ig and anti-CD Ig) was monitored by ELISA.

5   As expected from the results obtained from the same immunizations of the cows, no anti-CTB antibodies and anti-CD antibodies were detected in the milk of the goats after two immunizations via the IN route. Measurements of anti-CTB IgA (Figure 16) and anti-CTB IgG (Figure 17) in milk samples collected before the boost treatment showed no detectable levels. The same
10   result was observed for anti-CD (whole cell) IgA (Figure 18) and IgG levels (Figure 19). Two weeks after priming, the goats received boost treatments, which consisted of one 2 ml IM, one 2 ml IUDR and one 1ml LMF.
In case of the CD vaccine, an increase of anti-CD IgG levels in the milk was observed in 50% of the goats one week after the boost immunization (Figure
15   19). The anti-CD IgG secretion was evenly distributed among the two halves of the udder and the level of secretion was maintained for at least two weeks. Unlike the immediate IgG response, anti-CD IgA (Figure 18) responses (50% of the goats) were observed at least three weeks after the boost immunization. The anti-CD IgA secretion was also evenly distributed among both halves of
20   the udder. In comparison to the titers anti-CD of the standard (1000 units/ml), immunogen-specific IgA reached 20-25% of the standard levels.
In case of the cholera vaccine (Dukoral), an increase of anti-CTB IgG (Figure 17) levels in milk was observed in 50% of the goats starting from 1-2 weeks after the boost immunization. The anti-CTB IgA (Figure 16) responses
25   observed in 50% of the goats were slow to appear and could be detected more than a month later. As was observed with the CD vaccine, anti-CTB IgA and IgG secretion in the milk of the immunized goats was evenly distributed in the whole (two halves) udder. In comparison to the titers anti-CTB of the standard (1000 units/ml), immunogen-specific IgA reached >25% of the standard levels.
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In conclusion, these results demonstrate that the immunization method used previously in cows is also applicable to other mammals. There is a variation among goats in immune responses, however 50% of the animals showed immunogen-specific Ig levels in the milk after immunization. In all cases the
5   immunogen-specific IgA response is significantly higher than the IgG response. Both anti-CTB IgA and IgG responses observed in goats were significantly higher than anti-CD IgA and IgG responses. The immunogen-specific Ig response in the milk of immunized goats is not restricted to one half of the udder, i.e. the immunized half (right) is not the one that secreted the
10   highest Ig level (not shown). For cows the quarter specificity was observed in case of the anti-CTB IgA response. This dissimilarity can be explained by the different physiology of the udder of cow and goat.

As used herein the term IMA route and IUDR route both refer to intra-udder
15   immunization routes.

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BRIEF DESCRIPTION OF THE DRAWINGS

5   Fig. 1 is a graphic illustration of relative levels of anti-CTB IgA and IgG in colostrum of immunized cows, which were employed as the standard.

Fig. 2 is a graphic illustration of relative levels of anti-CD IgA and IgG in colostrum of immunized cows, which were employed as the standard.

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Fig. 3 is a graphic illustration of anti-CTB IgA responses (versus 1,000 units/ml of the standard) in the milk of cows after application of various immunization methods known in the art.

15   Fig. 4 is a graphic illustration of anti-CTB IgG responses (versus 1,000 units/ml of the standard) in the milk of cows after application of various immunization methods known in the art.

Fig. 5 is a graphic illustration of anti-CTB IgA responses (versus 1,000 units/ml
20   of the standard) in the milk of cows after application of immunization methods of the invention.

Fig. 6 is a graphic illustration of anti-CTB IgG responses (versus 1,000 units/ml of the standard) in the milk of cows after application of immunization
25   methods of the invention.

Fig. 7 is a graphic illustration of anti-CD IgG responses (versus 1,000 units/ml of the standard) in the milk of cows after application of immunization methods of the invention.

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Fig. 8 is a graphic illustration of anti-CD IgA responses (versus 1,000 units/ml of the standard) in the milk of cows after application of immunization methods of the invention.

5   Fig. 9 is a graphic illustration of relative anti-CD IgG responsiveness in milk after various routes of immunization.

Fig. 10 is a graphic illustration of relative anti-CD IgA responsiveness in milk after various routes of immunization.

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Fig. 11 is a graphic illustration of anti-CD IgA response (versus 1,000 units/ml of the standard) in milk of cows as the result of boosting via the LMF plus the IMA routes.

15   Fig. 12 is a graphic illustration of anti-CD IgG response (versus 1,000 units/ml of the standard) in milk of cows as the result of boosting via the LMF plus the IMA routes.

Fig. 13 is a graphic illustration of relative levels of total Igs in the milk of
20   immunized cows compared to those of unimmunized cows.

Fig. 14 is a graphic illustration of relative levels of anti-CTB IgA and IgG in colostrum of immunized goats, which were employed as the standard.

25   Fig. 15 is a graphic illustration of relative levels of anti-CD IgA and IgG in colostrum of immunized goats, which were employed as the standard.

Fig. 16 is a graphic illustration of anti-CTB IgA responses (versus 1,000 units/ml of the standard) in the milk of immunized goats after application of
30   immunization methods of the invention.

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Fig. 17 is a graphic illustration of anti-CTB IgG responses (versus 1,000 units/ml of the standard) in the milk of immunized goats after application of immunization methods of the invention.

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Fig. 18 is a graphic illustration of anti-CD IgA responses (versus 1,000 units/ml of the standard) in the milk of immunized goats after application of immunization methods of the invention.

10   Fig. 19 is a graphic illustration of anti-CD IgG responses (versus 1,000 units/ml of the standard) in the milk of immunized goats after application of immunization methods of the invention.